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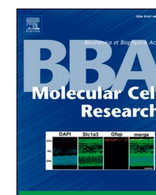

Review

The role of phospho-tyrosine signaling in platelet biology and hemostasis

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ABSTRACT

Platelets are small enucleated cell fragments specialized in the control of hemostasis, but also playing a role in angiogenesis, inflammation and immunity. This plasticity demands a broad range of physiological processes. Platelet functions are mediated through a variety of receptors, the concerted action of which must be tightly regulated, in order to allow specific and timely responses to different stimuli. Protein phosphorylation is one of the main key regulatory mechanisms by which extracellular signals are conveyed. Despite the importance of platelets in health and disease, the molecular pathways underlying the activation of these cells are still under investigation. Here, we review current literature on signaling platelet biology and in particular emphasize the newly emerging role of phosphatases in these processes.

1. Introduction

The human body has in the order of 10^{12} circulating platelets. Their average life span is 7–10 days, after which they are removed from the circulation and cleared by splenic or hepatic macrophages and hepatocytes. With a size of around 2–3 μm diameter, a discoid shape, and a minimal displacement volume coupled with a highly active cytoskeleton, platelets are well suited to vascular circulation. As the first responders to tissue damage, platelets are principle regulators of hemostasis. However, as suppliers of growth factors they also induce blood vessel formation and play a pivotal role in the wound healing and regenerative processes contributing to tissue repair. Additionally, a role for platelets in immunity is now emerging - having retained several of the functions associated with their myeloid origin, platelets have the ability to internalize environmental factors such pathogens through phagocytosis [1]. Binding of bacteria via toll like receptors may facilitate the recruitment and subsequent uptake of bacteria by granulocytes [2]. Platelets also contribute to adaptive immunity, by activating dendritic cells and enhancing their antigen presentation in the context of major histocompatibility complex (MHC)II molecules to T or B cells [3], while MHCI molecules taken up from serum can be used to present antigens to T-cells [4]. Thus, platelets are versatile cells which play an important role in human physiology. While a protagonist in hemostasis, excessive platelet activation is also a major cause of morbidity and mortality in western societies. It is therefore not surprising that platelets have become extensively investigated. Nevertheless, precisely how platelets become activated under physiological and pathophysiological conditions is still under investigation.

Despite the fact that platelets do not contain a nucleus and are hence incapable of transcribing new mRNA molecules, they do retain the capacity to synthesize their own functional proteins from mRNAs inherited from the megakaryocytes from which they derive. Alternative splicing and signal-dependent translation of the 3000–6000 platelet mRNA transcripts thus far identified may give rise to new proteins [5]. Furthermore, protein modification through functional proteasomes and ubiquitin complexes can take place. However, one of the most important post-translational protein modifications, allowing platelets to fulfil their highly dynamic and diverse functions [6,7], comes from protein phosphorylation. Reversible phosphorylation of proteins, catalyzed by phosphorylating kinases and dephosphorylating phosphatases, plays a role in almost every cellular aspect. Contact of platelets with extracellular ligands causes a fast and transient burst in kinase activity and a substantial augmentation of intracellular phosphorylation. However, although much is known regarding the kinases governing platelet functionality, comparatively little is known with regards to phosphatases in this context. In the early 90's, platelets were recognized to contain at least 6 independent phosphatases with activity towards actin-related proteins including myosin light chain (MLC), however these

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remained unnamed and were speculated to merely reduce platelet activation [8]. Subsequent studies have shown that platelets contain many more phosphatases, and that their role is not always so straightforward. Therefore, in this review, we will discuss the recent advancements in our knowledge regarding the phosphatases governing platelet plasticity in response to different stimuli.

2. Phosphorylation in platelets

Cellular communication depends on the binding of adhesion molecules, cytokines, chemokines or growth factors to ligand-specific receptors at the cellular membrane, ensuring amplification of the received information and execution of a specific cellular response. In this signaling cascade, activation or inhibition of enzymes is often determined by their phosphorylation status. Zahedi and colleagues investigated the phosphoproteome from healthy human platelets, and demonstrated that almost 280 proteins were phosphorylated on different sites, with 55% of these proteins related to signaling pathways and cytoskeletal dynamics [8]. Thus, phospho-signaling in platelets is evidently important, and a better understanding of these signaling events may provide insight into platelet biology in health as well as pathological conditions (Fig. 1). Protein phosphorylation is restricted to only a few amino acid residues, and according to their specificity, kinases and phosphatases are thus classified as being tyrosine, serine/threonine or dual specificity enzymes. Over 500 protein kinases are encoded by the human kinome, many of which are expressed in platelets. Although full functional characterization is mainly limited to a few main signaling pathways, our knowledge of these pathways is relatively well advanced [9–12]. In contrast, the human genome contains far fewer phosphatase genes, with a total of 107 protein tyrosine phosphatase (PTP) and ~ 30 serine/threonine phosphatases thus far identified [13]. PTPs can be further subdivided into four classes (I-IV), depending on their structural features and specificity. Class I consists of the classical PTPs (further subdivided into receptor PTPs and non-receptor type PTPs) and dual specificity phosphatases. Class II only contains the low molecular weight PTP (LMWPTP), while class III contains three CD25-like PTPs. In contrast to the cysteine-based PTPs in class I to III PTPs, Class IV contains aspartate-based PTPs.

Of the classical PTPs, sixteen were identified in platelets using a specialized PTP-proteomics approach [14], with several others identified by conventional proteomics, making a total tally of 10 receptor-like and 10 non-receptor PTPs present in platelets [15,16]. Serine/threonine phosphatases, including PP1, PP2, PP3, PP6 have also been identified in platelets [17–20], as well as the dual specificity phosphatases DUSP3 and MTM1 [18]. In addition, the novel histidine phosphatase TULA-2 has been shown to be present in platelets [21], as is LMWPTP [22]. We summarized the expressed phosphatases reported in platelets of the tyrosine, serine-threonine and dual-specificity phosphatases in Fig. 2. Lipid phosphatases, while not the main topic of this review, have also been shown in platelets [23]. Over the past few years, more and more studies have revealed that phosphatases are relevant modulators of platelet function, although the underlying mechanisms are still poorly understood. It is of interest to note that *pan* protein tyrosine phosphatase activity is increased in platelets in response to collagen stimulation,

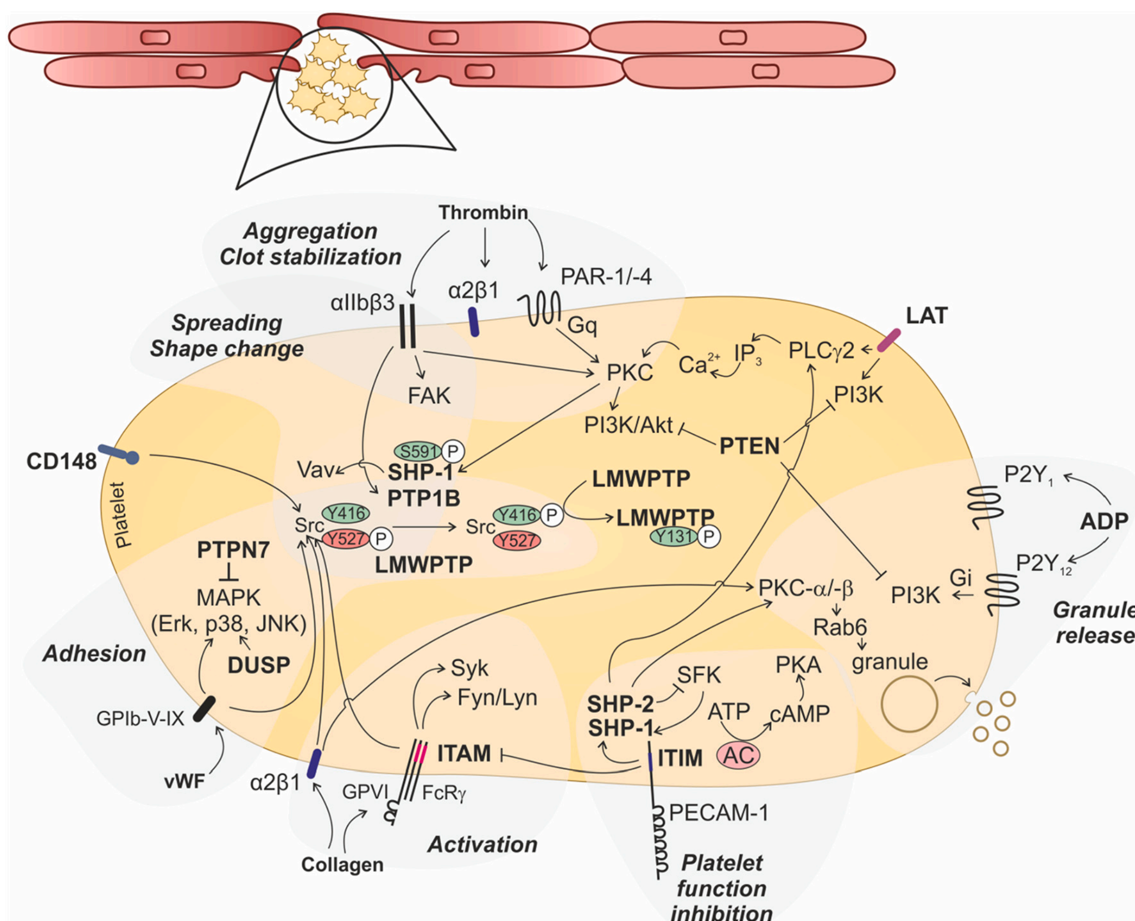

Fig. 1. Platelet function. Platelet activation is modulated by the interaction of several agonists with specific receptors. After the activation through diverse stimuli, platelets change their shape to spread and expose adhesion molecules, which supports adherence and triggers granule release, further amplifying the aggregation process. Following the platelet activation, platelets releases pro-inflammatory factors for wound healing, tissue regenerations and angiogenesis by transporting and delivering factors.

Tyrosine phosphatase

Phosphatases expressed in platelets

Class I

Classical

Receptors

PTPRA, PTPRB, PTPRC, PTPRG, PTPRK, PTPRO, PTPRT [18, 141, 142]
PTPRJ* [107]

Non-receptors

PTP4A, PTP4A1, PTPN12, PTP18 [18, 141, 142]
PTP1B/PTPN1*, PTPA2*, SHP1/PTPN6*, PTPN7*,
MEG2/PTPN9*, SHP2/PTPN11* [18, 22, 90, 92, 140-144]

Dual-specificity

DUSP3* [126]
MTM1 [18]

Class II

LMWPTP* [22]

Serine threonine phosphatase

PPP

PP3, PP6 [18]
PP1*, PP1C α*, PP1C δ*, PP1M*, PP2A* [118, 135-139]

*Functional analysis reported in platelets

Fig. 2. Protein phosphatases described in platelets. Summary of protein phosphatases described in platelets on expression level, and functional analysis described [92,135–144].

suggesting their role in adhesive processes (Fig. 3). However, in contrast to collagen, thrombin stimulation reduces total PTP activity (Fig. 3), demonstrating ligand-specific regulation of phosphatase activity in platelets. Below, we summarize the ligand-receptor-signaling events involved in hemostasis and review what is currently known regarding the best described of these phosphatases in platelet activity.

3. Platelet receptors, their ligands and hemostasis

In order to perform their functions, platelets carry an array of membrane receptors allowing them to respond to various extracellular ligands. Of the extended family of integrins, platelets carry six: α2β1 (CD49b/CD29), αvβ1 (CD49e/CD29), α6β1 (CD49f/CD29), αLβ2 (CD11a/CD18), αIIbβ3 (CD41/CD61), and αvβ3 (CD51/CD41) [20,24]. Of these, arguable the best studied are the highly expressed αIIbβ3 [20], which is recognized by fibrinogen, von Willebrand factor (vWF) and the extracellular matrix components fibronectin and vitronectin, and the collagen receptor α2β1 (otherwise known as glycoprotein GPIa/IIa). Collagen is additionally recognized by GPVI. The second most abundant platelet receptor is the glycoprotein receptor complex GP1b-IX-V, which primarily binds to vWF, but also recognizes thrombin, P-selectin, and clotting factors XI and XII [25]. Thrombin is also able to stimulate platelets via the protease activated receptor (PAR)1 and PAR4 (see Fig. 1).

The first step towards thrombus formation is the deceleration of platelets through binding of their GPIb-V-IX receptor complex to vWF captured onto collagen fibrils exposed at the sub-endothelial matrix lining of the vessel upon injury [26,27]. This interaction is stabilized by subsequent binding of α2β1 to collagen and αIIbβ3 binding to vWF/fibrinogen [20,27,28]. After formation of a monolayer of platelets attached to the vessel wall, homotypic platelet interactions mediated by GPIb-V-IX and αIIbβ3 binding to their ligands causes platelet aggregation. Subsequent adhesion-dependent signals, including GPVI binding to collagen, triggers activation of the platelets [29], which is further extended by locally generated or secreted factors like thrombin, ADP, epinephrine and thromboxane A2 (TxA2). At this stage, the activated platelet changes its morphology from a discoid shape to a flattened shape with lamellipodia, thus increasing their surface area and providing a scaffold for other aggregating platelets (Fig. 1A) [30–32]. In the last steps of platelet activation, conformational changes in integrin αIIbβ3 resulting in enhanced affinity for its ligands, allows fibrinogen to act as a bridging molecule, tethering platelets together [33,34]. The inside-out signaling required to initiate these conformational changes appears to be mediated largely through thrombin-mediated signaling via PAR1, and depends on actin-binding intermediator proteins such as talin, kindlins and the ezrin-radixin-moesin (ERM) family of proteins [35,36]. In addition to forming a platelet plug during primary hemostasis, platelets contribute to secondary hemostasis (the coagulation cascade) through their release of coagulation Factors V, XI, and XIII, prothrombin and fibrinogen, all of which feed into the coagulation cascade. This series of catalytic steps culminates in the cleaving of fibrinogen by thrombin to form adhesive monomeric fibrin fibrils, which further

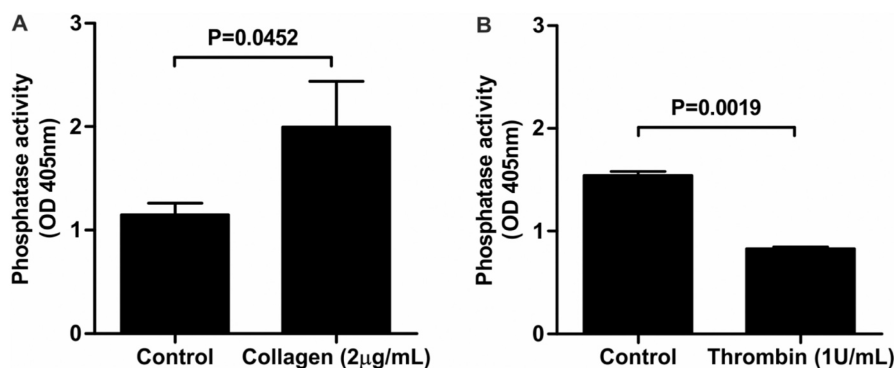

Fig. 3. Total tyrosine phosphatase activity assay in stimulated platelets. After signed informed consent was obtained (Ethical committee Project NL66029.078.18 approved by Erasmus MC medical and ethical committee), venous blood from healthy donors ($n = 3$) (all of them drug-free) was collected into conical plastic tubes containing 3.8% trisodium citrate 1:10 (*v/v*). Whole blood was centrifuged at 1500 rpm, 10 min, 22 °C, and Platelet-Rich Plasma (PRP) was collected. NaCl (0.9%) was used to wash the platelets. Washed platelets were incubated with a final concentration of 2 μg/mL Collagen or 1 U/mL Thrombin for 10 min. All experiments were performed using 200–300 x 10^3platelets/μL. Platelets were lysed with 100 μL of lysis buffer (20 mM HEPES, pH 7.4 with 2.5 mM $MgCl_2$, 0.1 mM EDTA) on ice for 2 h. After clarifying by centrifugation, protein-normalized platelets extracts were incubated in acetate buffer and the enzyme activity was assessed as followed: reaction medium (100 μL) contained 100 mM acetate buffer, 5 mM *p*-nitrophenyl phosphate (pNPP). After 60 min, at 37 °C and under agitation (600 rpm) the reaction was stopped by adding 100 μL 1 M NaOH. The absorbance was measured at 405 nm (spectrophotometer - BioRad, California, USA). Statistical analysis was performed using t-student (paired, 95% confidence intervals, one-tailed) using GraphPad (GraphPad Inc., version 5.0, California, USA) - (A.V.S. Faria, unpublished data).

constructs stable bridges between platelets [37].

It is of interest to note that platelets carry many components capable of enhancing their own activation. Among others, the fibrin precursor fibrinogen, GPVI, αIIbβ3, P-selectin, and bioactive molecules such vWF, are contained within α-granules [38,39]. Furthermore, dense-granules, lysosome-related organelles with a cargo profile that is dominated by smaller molecules, contain serotonin, ADP/ATP and Ca^{2+} [40]. Exocytosis of these granules can be triggered by specific platelet agonists and enhances expression of granule-specific membrane phospholipids, glycoproteins and integrins. Many of these membrane components play an essential role in platelet adhesion and aggregation, and their release can therefore enhance platelet activation cascades. Furthermore, as platelets themselves carry receptors for many of the vesicle components, including ADP receptors $P2Y_1$, $P2Y_{12}$ and the ATP receptor $P2X_1$, release of these factors allows autocrine activation [41].

4. Signaling via platelet receptors during hemostasis and thrombus formation

4.1. G-protein coupled receptor signaling

GPCRs receptors are coupled to heterotrimeric G-proteins, Gα, Gβ and Gγ, of which in particular the type of Gα subunit determines the signaling outcome. Four main Gα proteins can be distinguished: Gαq, Gαs, Gαi and $G\alpha_{12/13}$. Platelet Gαq receptors include the receptors for thrombin [42], TxA2 and the P_2Y_1 ADP receptor. Binding of a Gαq-coupled receptor leads to activation of phospholipase C, which in turn cleaves the membrane-bound inositol lipid phosphatidylinositol biphosphate (PIP2) to diacylglycerol (DAG) and inositol 3-phosphate (IP3) [43]. Subsequent binding of IP3 receptors at the endoplasmic reticulum leads to release of intracellular Ca^{2+} stores, while DAG binds and activates the serine/threonine Protein Kinase C (PKC), together leading to further intracellular signal transduction and cellular responses. Gαs and Gαi proteins act by stimulating or inhibiting the enzyme adenylate cyclase (AC), respectively. AC activity triggers the formation of the second messenger cyclic-AMP (cAMP) resulting in activation of cAMP-dependent Protein Kinase A (PKA), a potent inhibitor of platelet function [44]. While prostaglandins and adenosines increase cAMP levels via Gαs, the thrombin receptor PAR1, as well as the ADP receptor $P2Y_{12}$, signal through Gαi (as well as Gq), thereby reducing activation of the serine/threonine PKA kinase [45]. Different isoforms of PKA exist, which may localize to different cellular compartments and play different roles in platelet activation [46] suggesting that PKA signaling in platelets is complex and multifunctional [45]. Lastly, signaling via $G\alpha_{12/13}$ occurs via PAR4 and the TxA2 receptors, and activates cytoskeletal rearrangement and platelet shape changes [47].

Phosphotyrosine profiling shows that total cellular tyrosine phosphorylation increases upon stimulation with ATP, and is near completely abolished by concurrent treatment of cells with PKA-activating prostacyclin analogue iloprost [48]. This suggests that kinase activity does not indiscriminately result in enhancement of platelet functions, as is often thought. Indeed phosphorylation of $GP1b\beta^{Ser166}$ by PKA can reduce its binding to vWF under flow conditions [49]. Large scale phosphoproteomics confirmed that inhibition of platelet functions through stimulation with prostacyclin (activating cAMP/PKA signaling) is indeed accompanied by upregulation of phosphorylation of several kinases, including $GRP2^{Ser587}$, Src^{Tyr530}, $VASP^{Ser157}$, $VASP^{Ser239}$, $GSK3\alpha^{Ser21}$, $GSK3\beta^{Ser9}$, $Zyxin^{Ser142/143}$, $Filamin\text{-}A^{Ser2152}$, $LASP^{Ser14}$. However, many of these phosphorylation reactions reduce activity of the associated protein, which may in part account for the inhibition of platelet functioning [50–52]. In addition, phosphorylation of 3 phosphatases, namely $PTPRJ^{Ser1311}$ (increased), $CTDSPL^{Ser32}$ (decreased), and $PTPN12S^{er332}$ (decreased) were affected by prostacyclin stimulation. To what extent these contribute to inhibition of platelet signaling in response to Gαs signaling remains to be established (for more details on PTPRJ, see below) [44].

4.2. Tyrosine kinase (associated) receptor signaling

The main tyrosine kinase-associated receptors involved in platelet activation are GPIb-IX-V and GPVI. Neither of these contain intrinsic kinase activity; instead, they rely on a family of protein-tyrosine kinases (PTKs) that are either associated with or in close proximity to their cytoplasmic tails, to transmit signals. For GPVI, this is provided by coupling with Fcγ-receptors, which have an intracellular domain containing an immunoreceptor tyrosine activation motif (ITAM). Upon ligand binding, ITAM motifs become phosphorylated by the Src family kinases (SFK, including Src, Syk, Fyn, Lck, Btk, Fgr, Lyn, Yes), which results in tyrosine phosphorylation of the membrane adaptor protein LAT (linker of activation of T-cells), in turn activating multiple signal transduction pathways including PLCγ and the lipid kinase phosphatidyl-inositol 3-OH kinase (PI3K) [53,54]. For GPIb/IX/V, the associated tyrosine kinase appear to be less well known, and signaling could be either via FcγRII, directly through Src, or via PI3K [54]. It has also been suggested that FcγRII-ITAM signaling contributes to outside-in signaling of integrins, with in particular Fyn and Syk contributing to phosphorylation of αIIbβ3 integrin, leading to PLCγ activity [47,55]. Other tyrosine kinase receptors present on the surface of human platelets include the thrombopoietin receptor (or c-MPL), the TAM family (Tyro3, Axl, and Mer), ephrins and the IGF-1 receptor [54].

Downstream signaling of tyrosine kinase (associated) receptors is complex, and in platelets appears to depend to a large extent on SFK activity (for review see [10]). vWF interaction with GPIb-V-IX leads to activation of SFK as well as Phospholipase A2, initiating platelet activation by changing its cytoskeletal arrangement [10,53]. Collagen stimulation of the receptors αIIbβ3, GPIb, GPVI and FcγR also results in SFK activation [53]. Furthermore, in platelets activated by thrombin, Ca^{2+}-mediated activation of SFK results in activation of Pyk (a member of the focal adhesion kinase [FAK] family) [56], both described as master regulators of cytoskeletal remodeling in response to thrombin, showing that GPCR signaling also converges on tyrosine phosphorylation signaling [57,58]. This is further demonstrated by the converse fact that collagen stimulation results in Ca^{2+}-dependent activation of PKC-α/β which promotes α-granule release [59]. Activation of SFK in platelets leads to activation of other intracellular kinases, including PI3K/protein kinase B (otherwise known as Akt). For instance, α2β1 and αIIbβ3 activate SFK and PLC signaling, which coordinates PI3K/Akt activation [9,60]. PI3K was also associated with platelet aggregation in response to ADP stimulation [61,62], as well as thrombin [58]. Downstream of Src-PI3K signaling, Akt phosphorylation was confirmed to play an important role in collagen- and ADP-induced platelet aggregation [61]. In addition to Src and PI3K signaling, members of mitogen activated protein kinase (MAPK) family (e.g. ERK and p38) have generally been considered positive modulators of platelet function. Binding of vWF or thrombin to GPIb-IX-V results in activation of integrin αIIbβ3, an example of inside-out-signaling, and this process depends on both PKC and ERK activities [63]. Activated ERK is described to play an important role in platelet aggregation, morphological changes and clot retraction [64]. p38 is also known to be activated by Src phosphorylation in response to collagen or thrombin. However, the role of ERK and p38 are dissimilar, with p38, but not ERK, mediating collagen-induced adhesion under static conditions and ERK regulating collagen/GP1b-vWF interactions and p38 mediating collagen-αIIβ1 interactions under shear conditions [65,66]. The principal functions coordinated by p38 are cytoskeleton remodeling and Ca^{2+} release, and consequently, amplification of platelet aggregation [67].

The general kinase receptor-induced pathways described here are common among many different cell types, and phosphatases modulating these pathways have been identified in several cellular settings. Below, we describe in more detail what is known about these phosphatases signaling in the dynamic modulation of phosphoprotein content in

platelets.

5. Phosphorylation and dephosphorylation in platelet shape change and degranulation

Actin cytoskeletal rearrangement is required for platelet shape change, contraction, degranulation and aggregation. Serine phosphorylation of MLC, causing the formation of cross-bridges between myosin and actin, plays an important role in these processes [68]. Activation of MLC can be achieved through both Gαq and $G\alpha_{12/13}$ pathways. For instance, Gαq/PLCβ activation through thrombin modulates platelet shape changes via IP3-Ca^{2+} signaling [69] Early studies already indicated that in addition to PLC/PKC signaling, thrombin-mediated platelet aggregation relies on activation of tyrosine- and serine/threonine phosphatases [70]. While the exact phosphatases remained to be elucidated at that time, later studies demonstrated that activation of diverse PKC isoforms inhibits the MLC phosphatase (MLCP), thereby allowing MLC phosphorylation and stimulation of platelet granule secretion [71,72]. Activation of $G\alpha_{12/13}$ by for instance TxA2 also results in phosphorylation of MLC and shape changes, but in this case depends on Ca^{2+}-independent signaling, including Src and Rho-kinase activation [70,71], which in turn phosphorylate and inactivate MLCP. Similarly, PAR4-mediated $G\alpha_{12/13}$ activity activates Pyk2, regulating platelet aggregation and dense granule release [72], and MLCP appears to be the target of Pyk2 signaling through this G-protein [72]. In contrast, prostaglandin-Gαs-activated PKA activity inhibits the Rho kinase and enhances MLCP activity, thereby attenuating the actin cytoskeleton rearrangements required for platelet shape changes [73]. In addition to GPCR signaling, collagen/αIIb mediated degranulation has also been linked to MLC phosphorylation [68], although in this case via ERK-mediated activation of the MLC kinase. Thus, a complex series of activating and inactivating phosphorylations of both kinases and phosphatases converge on MLC-mediated modulation of the platelet cytoskeleton.

MLC-independent pathways of platelet shape change/degranulation have also been described to rely on phosphatase activity. Ca^{2+}-dependent but PKC-independent platelet dense granule release induced by thrombin was shown to be mediated via the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, through activation of the Rho GTPase Rac and dephosphorylation of the actin modulator cofilin [74,75]. The lipid phosphatase SHIP1 also modulates dense granule release. Collagen-GPVI stimulation of platelets results in SFK-mediated activation of SHIP1, which associates with PKC-δ and inhibits granule release. In contrast, thrombin-stimulated PKC-δ activity does not coincide with SHIP1 association and stimulates dense granule secretion [76]. The complexity of PKC signaling is further demonstrated by the fact that (as mentioned above) α-granule release in response to collagen is stimulated by PKC-α/β, while collagen-induced PKC-θ negatively modulates α-granule degranulation, and PKC-δ has no effect [59]. In addition, tyrosine kinase receptors, including Axl and Tyro3 mediated Syk/PLCγ signaling also play important role in platelet activation, especially in α-granule release and spreading, although the role of phosphatases in these signaling cascades remains unstudied [54].

In addition to the actin cytoskeleton (the microfilaments), microtubules play an important role in platelet physiology, in particularly in maintaining platelet discoid shape, regulating intracellular transport of vesicles and their degranulation [77]. α-Tubulin, the building blocks of microtubules, undergo many post-translational modifications in activated platelets, resulting in reorganization of the microtubule cytoskeleton and contributing to platelet aggregation [78,79]. Tubulin is phosphorylated in unstimulated platelets and loses phosphorylation levels upon stimulation with agonists [80,81] such as thrombin or collagen (although this was disputed by others) [82], however it is unclear to what extent phosphatases contribute to this process. Nevertheless, MLC-independent PP1 and PP2-mediated dephosphorylation of a 90 kDa target was shown to contribute to both actin and microtubule dynamics in platelets [83], although in this case the target was not identified. Taken together, both microfilament and microtubule dynamics in platelets are modulated by phosphatase activity although the direct extent to which phosphatases are involved requires further elucidation.

6. The role of phosphatases in hemostasis and thrombus formation

6.1. ITIMs, GPCRs and SHP

Platelet activation via ITAM motif-coupled receptors is counteracted to large extent by immunoreceptor tyrosine-based inhibitory domain (ITIM)-containing receptors. The best described of these in platelets are PECAM-1, which mainly inhibits collagen-mediated activation of the GPVI-FcRγ-chain complex, and G6b-B, which reduces signaling from GPVI-FcRγ as well as the hemiITAM-containing receptor for podoplanin; CLEC2. In addition to an ITIM motif, PECAM-1 and G6b-B contain an immunoreceptor tyrosine-based switch motif (ITSM) [84]. Phosphorylation of both ITAM and ITIM/ITSM by SFKs creates docking sites for SH2-domain containing proteins. But while for ITAM this results in recruitment of protein-tyrosine kinase Syk and downstream activation of (tyrosine) kinase signaling and Ca^{2+} release, ITIM/ITSMs recruit the SH2-domain containing phosphatases SHP1 (i.e. PTP1C, PTPN6) and SHP2 (i.e. PTP1D, PTP2C, PTPN11), which is associated with inhibition of platelet function [85]. SHP2 may directly dephosphorylate the ITAM receptor itself (as was shown for CLEC-2 in the case of G6b-B), but also may target the kinase Syk [84–86]. Additionally, it has been suggested that PECAM-1 itself may be a target (although the role for this in platelets is unclear) and that recruitment of PI3K to SHP1 prevents its activation by molecules associated with LAT (although this appears to be independent of its phosphatase activity [87–89]. Under collagen stimulation, the formation of reactive oxygen species causes transient oxidation of SHP2, which inactivates its phosphatase activity and allows activation of Syk, Vav and Btk in the LAT complex, resulting in platelet activation and degranulation [90]. It should be noted that association of SHP2 with the non-classical ITIM-bearing receptor TREM-like 1 (TL1), actually enhances α-granule release, adding a layer of complexity to SHP signaling [91].

The role of SHP1 and SHP2 has also been investigated in non-ITIM-mediated signaling in platelets. As already alluded to above, GPCR signaling activates kinase signaling and phosphorylation patterns which are also subject to phosphatase regulation. One study showed that PKC-mediated phosphorylation of $SHP1^{Ser591}$ in response to thrombin-PAR1/4 stimulation inhibits SHP1 activity, which subsequently increases phosphorylation of its substrate, the guanine-nucleotide exchange factor Vav, a regulator of cytoskeletal rearrangement [89]. Indeed, overall, the general consensus appears to favor a negative modulatory role for SHP phosphatase activity in platelet adhesion [90]. However, several studies indicate that the role of SHP phosphatases may be more complicated. $SHP1^{Tyr536}$ and $SHP1^{Tyr564}$ phosphorylation in response to thrombin were shown to activate SHP1 enzymatic activity [93], independently from integrin and PKC signaling [94]. As the Src kinase is a direct substrate of SHP1, this would paradoxically dampen thrombin-mediated responses. However, some studies showed that this may be a late event during thrombin signaling (upon 45 min stimulation), and therefore present a means to prevent over-activation of thrombus formation [95]. Furthermore, by targeting the negative tyrosine regulatory site of Src kinase, SHP1 activity may actually enhance Src activity and platelet function [96]. Additionally, it was demonstrated that Gαq-mediated signaling causes rapid (within seconds) degradation of a protein complex which keeps resting platelets inactivated, and this process is dependent on phosphorylation and activation of SHP1 [97]. Overall, both positive and negative roles for SHP1 have been described in platelets and the exact role for SHP1 in platelet function therefore remains elusive [97,98]. Experiments in knock-out mice have

demonstrated that megakaryocyte-specific *Shp1* deficiency leads to reduced platelet spreading, while *Shp2* deficiency increases platelet spreading on fibrinogen, demonstrating opposite effects of these phosphatases on at least some platelet functions [99]. Consistently, in vivo thrombosis was only reduced in *Shp1* but not *Shp2* knock-out mice [99]. Both gain-of-function and loss-of-function mutations of *SHP2* are found in humans: gain-of function mutations as seen in patients with Noonan syndrome (often associated with bleeding) cause reduced collagen-induced (but not thrombin or ristocetin-induced) platelet aggregation, while loss-of-function of SHP2 in patients with Noonan syndrome with multiple lentigines show normal aggregation, but increased collagen-adherence under shear stress [100]. This would suggest that SHP2 negatively modulates collagen-induced platelet reactions. Indeed, *Shp2*-deficient mouse platelets form more stable thrombi on collagen under shear stress as compared to wild type platelets [101]. However, while Shp2 downregulates αIIbβ3 outside-in signaling and reduces TxA2-mediated dense granule release, no effect of *Shp2*-deficiency was seen on collagen, thrombin, or ADP-mediated aggregation or dense granule release [101]. On the other hand, stimulation of platelets with thrombin (via PAR4) results in association of SHP2 with PI3K and Pyk in human platelets, which together seems to stimulate platelet aggregation, despite the presence of phosphatase activity in this complex. Thus, SHP2 activity may either control the thrombin-induced signal or actually enhance it [102].

6.2. Positive modulation of SFK via CD148 and PTP1B

One of the first transmembrane PTPs identified in platelets is the receptor-like protein-tyrosine phosphatase (PTP) CD148, also known as PTPRJ, which is expressed in all hematopoietic cells. It is known to dephosphorylate PLCγ1 and LAT, thereby inactivating signaling through the T-cell receptor CD3 in T-cells, while dephosphorylation of SFKs contributes to neutrophil migration [103,104]. It is tempting to speculate that CD148 may contribute to the immunological functions of platelets as well. While one of the main substrates of this phosphatase is the platelet derived growth factor receptors (PDGFR), in platelets this phosphatase is best known for its ability to modulate activity of the SFKs [105]. Knockdown of CD148 in mouse models was shown to increase Lyn and Src phosphorylation at their inhibitory sites, while reducing phosphorylation in their activation loops. In turn, this resulted in impaired platelet spreading on fibrinogen, suggesting a requirement of this phosphatase in integrin αIIbβ3 signaling. GPVI-dependent platelet aggregation and ATP secretion were also dependent on CD148 activity. Thrombin-induced aggregation and ATP secretion as well as CLEC-2-mediated platelet activation were only partly affected by CD148 knock-down, while ADP-induced responses were not affected at all, showing selective requirement of this phosphatase by specific platelet agonists [106]. Knockdown of CD148 reduces thrombosis in mice, suggesting that CD148 inhibition may be beneficial for to limit or treat thrombotic events [107]. In humans, it has recently been shown that loss-of-function mutations in CD148 cause a form of hereditary thrombocytopenia, with patients showing reduced pro-platelet formation as well as reduced GPVI-mediated Src activation and platelet responses [106]. Thus, the general consensus is that CD148 contributes to platelet activation by releasing inhibitory phosphorylation on SFK. However, a recent study suggests that CD148 may also reduce phosphorylation of SFKs at their activation loop. However, this is likely required to limit platelet over-activation, and indeed over-activation of SFK signaling may provide a negative feedback loop [108].

While CD148 was shown to be essential for platelet activity, full aggregate formation also requires the phosphatase PTP1B, another positive modulator of SFK [109]. Platelet expression of the non-transmembrane PTP1B (also known as PTPN1) was already described decades ago. This phosphatase can become activated upon proteolytic cleavage by calpain in response to αIIbβ3 integrin stimulation [110]. This in turn reduces the FcyRIIa-mediated phosphorylation of LAT, resulting in cytoskeletal rearrangement and irreversible platelet aggregation [111]. This suggests that PTP1B activity is required for full platelet responses, and indeed, PTP1B deficient platelets fail to form full aggregates on collagen [107]. Upon fibrinogen stimulation of platelets, PTP1B is recruited to interact with αIIbβ3 and promotes Src kinase activation by dephosphorylation of its inhibitory site [107,112]. This results in αIIbβ3 outside-in signaling and adhesion of platelets to fibrinogen. These functions are very similar as those reported for CD148, but while CD148 appears to regulate SFK more generally, PTP1B involvement is more specific to αIIbβ3 integrin signaling [105,113]. It should be noted however, that calpain knockout mice were described to exhibit increased platelet PTP1B levels as well as activity, which was associated with reduced thrombin-induced platelet aggregation, showing that PTP1B signaling is perhaps more complex [114]. While knock-out of calpain resulted in reduced in vivo thrombosis in mice, PTP1B knock-out mice showed similar occlusion times as wild type mice [114]. It should be noted however, that these were not conditional knock-out models. Interestingly, myeloid-specific knock-out of PTP1B from macrophages did reduce atherosclerosis in a mouse model [115].

Crosstalk between PTP1B and the serine/threonine phosphatase PP2A has also been shown. While PTP1B-mediated Src activation is required for αIIbβ3-mediated platelet adhesion, PP2A inhibits this process, adding a layer of regulation and complexity [116]. Indeed, serine phosphatases may contribute to tyrosine phosphorylation patterns in platelets [117]. For instance, αIIbβ3-activated PP2A inhibits ERK signaling in response to ATP [118]. Thus, not all phosphatases are alike and it is their concerted actions that decide platelet fate.

6.3. LMWPTP is activated upon collagen-induced signaling

The presence of LMWPTP was first described in platelets by Mancini and colleagues as late as 2007 [119]. They showed that while this phosphatase is less abundant in platelets as compared to PTP1B, it is nevertheless functional in causing dephosphorylation of the FcγRII receptor ITAM motif, as well as LAT, causing inactivation of Syk and PLCγ and inhibition of platelet functions. Interestingly, despite the fact that Scr kinases have been shown to be an important target for LMWPTP in other cell types [119–121], Src phosphorylation in platelets in response thrombin, convulxin or FcγRIIA clustering did not appear to be affected by LMWPTP in the study of Mancini et al. [119]. On the other hand, another study showed that collagen induces LMWPTP, PTP1B and Src kinase activity, and that inhibition of this phosphatase activity is associated with reduced Src activity and inhibition of platelet aggregation [22]. In addition, LAT is also a substrate for the kinases Syk and ZAP70, both of which are activated by LMWPTP [122,123]. Thus, the end result of LMWPTP activity in platelets may be activation rather than inactivation of platelet signaling, depending on the platelet agonist. While LMWPTP may play an important role in platelet activation, the molecular contribution of this phosphatase remains poorly understood.

6.4. Other tyrosine phosphatases

Very limited information is available concerning the other phosphatases expressed in platelets. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a well-known tumor suppressor, which, when down-regulated or mutated, is correlated to cancer development and obesity. In platelets, a function for PTEN was described by Weng and colleagues (2010) [124] showing that platelets deficient for PTEN demonstrate increased collagen-induced aggregation and ATP secretion, as a result of enhanced activation of the PI3K-Akt pathway [123]. Thus, knockdown of PTEN reduced bleeding times in mice [124]. In contrast, knockdown of the protein tyrosine phosphatase PTPN7 (or hematopoietic protein tyrosine phosphatase (HePTP)), which acts as a negative modulator of ERK, regulating GPCR-signaling rather than GPVI signaling, only modestly affected bleeding times [125]. However, time to death in a pulmonary thromboembolism model was

enhanced upon PTPN7 knock-out in this study. ERK is also targeted by the Vaccinia H1-related (VHR) phosphatase, or dual-specificity phosphatase (DUSP) 3. In vivo knock down of DUSP3 in mice, as well as its chemical inhibition, causes a decrease of platelet aggregation efficiency, and reduces microvessel occlusion in thrombosis models [126]. Thus, conflicting results are seen upon knock-down of PTPN7 or DUSP3 despite partial overlap in ERK modulation and in their signaling activity.

6.5. Targeting the platelet-expressed phosphatases: perspective

Investigation of the contribution of tyrosine phosphatases in platelets will likely emphasize their importance in diverse diseases, such cardiovascular, cancer, metabolic diseases [127,128]. The next step may, therefore, be to focus on the development of better inhibitors of phosphatases. Unfortunately, the search for phosphatase inhibitors is limited by several factors, including lower selectivity and bioavailability [129]. Protein tyrosine phosphatases are characterized by a PTP motif: (V/H) CX5R, which is part of the phosphate-binding loop (P-loop) and makes it so complex to evaluate appropriate and selective inhibitors [130]. In addition, the presence of many holo-enzymes makes it difficult to produce selective (non-toxic) inhibitors for some (serine/threonine) kinases [131]. While many phosphatase inhibitors exist which are readily used in in vitro settings, many of these show poor selectivity, and none are used in the clinic thus far [132]. Nevertheless, with more and more evidence suggesting that phosphatases play a stronger role in platelet hyperactivity than previously anticipated, exploring this class of enzymes as druggable targets could present a step forward in the treatment of platelet-related diseases [133]. With this being widely recognized, efforts are now being made to develop novel discovery strategies to identify selective phosphatase inhibitors [132,134]. In addition, phase I trials with PP2A inhibitors for the use in cancer therapy have been completed, and show favorable toxicity profiles and efficacy. To what extent inhibition of phosphatases might modulate platelet responses in a clinical setting remains largely unexplored. Interestingly alternative opportunities to inhibit phosphatase function may exist. Some phosphatases, including SHP2 and LMWPTP are inactivated by oxidation by reactive oxygen species (ROS) production. For instance, collagen-induced ROS limits SHP2 activity, and treatment of platelets with the ROS scavenger Reversatrol prevents SHP2 activation, allows dephosphorylation of key signaling molecules such as Vav1, Btk and PLCγ, reduces aggregation [91,102], suggesting that ROS scavengers could play a role in the treatment of thrombovascular diseases. Recently, we demonstrated that targeting LMWPTP and PTP1B (using 3-bromopyruvate and CinnGEL, respectively) seems to disturb platelet function through Src inhibition. However, SHP1/2 inhibition (using NSC87887) did not affect platelet function (100 μM) [22]. Furthermore, our unpublished data also suggest that Trodusquemine (MSI-1436) and Claramine (both PTP1B inhibitors), while modulating p38 and Src signaling, showed little effect on platelet aggregation. These data emphasize the need for better, more selective inhibitors, in order to exclude off target effects.

7. Conclusions

Platelets play an important role in many physiological processes, including hemostasis, wound healing and immunity. Impaired platelet function may contribute to several diseases, with over-activation of platelets having been related to, for instance, cardiovascular diseases and their complications. Thus, the elucidation of the molecular mechanism governing platelet functionality in health and disease may improve the treatment of such diseases. The role of phosphatases in these processes is slowly gaining attention, but their roles are as complex as the multitude of signaling events regulating platelet functions themselves. Both positive and negative modulatory roles for these enzymes have been described, and it is clear that selectivity towards agonists exists for these proteins in platelets. However, differences in agonists used, read-outs for platelet reactivity and methods of studying phosphatase activity and their downstream targets hamper interpretation of the limited experimental evidence. Thus the role of phosphatases in platelet biology warrants further investigation. A better understanding of these phosphatases, the way they themselves are regulated and the pathways they modulate, may contribute to the discovery of new potential pharmacological targets.

CRediT authorship contribution statement

AVSF carried out the experiment; AVSF, SSA and GMF original writing; MPP and CVFH revising.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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